EXHIBIT A

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Protein aggregation and neurodegenerative disease

Christopher A Ross & Michelle A Poirier

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and prion diseases are increasingly being realized to have common cellular and molecular mechanisms including protein aggregation and inclusion body formation. The aggregates usually consist of fibers containing misfolded protein with a β-sheet conformation, termed amyloid. There is partial but not perfect overlap among the cells in which abnormal proteins are deposited and the cells that degenerate. The most likely explanation is that inclusions and other visible protein aggregates represent an end stage of a molecular cascade of several steps, and that earlier steps in the cascade may be more directly tied to pathogenesis than the inclusions themselves. For several diseases, genetic variants assist in explaining the pathogenesis of the more common sporadic forms and developing mouse and other models. There is now increased understanding of the pathways involved in protein aggregation, and some recent clues have emerged as to the molecular mechanisms of cellular toxicity. These are leading to approaches toward rational therapeutics.

Neurodegenerative diseases and pathology

All of the diseases discussed here involve selective neuronal vulnerability with degeneration in specific brain regions, and deposits of abnormal proteins in neurons and other cells or extracellularly[1–9] (see **Table 1** and **Figure 1**). This review will consider mechanisms of protein misfolding and aggregation in relation to disease pathogenesis, along with therapeutic implications.

Huntington's disease. HD is a progressive neurodegenerative disorder caused by expansion of a CAG repeat coding for polyglutamine in the N terminus of the huntingtin protein. Because it is caused by a mutation in a single gene, HD has emerged as a model for studying neurodegenerative disease pathogenesis. There is a remarkable threshold effect, in that polyglutamine stretches of ≥36 in huntingtin cause disease, whereas ≤35 do not. Within the expanded range, longer repeats cause earlier onset. There is a striking correlation between the threshold for aggregation *in vitro* and the threshold for disease in humans, consistent with the idea that aggregation is related to pathogenesis[10,11]. Inclusions containing huntingtin are present in regions of the brain that degenerate. However, the neurons with inclusions do not correspond exactly to the neurons that degenerate. For instance, inclusions are present in the striatum, which is most affected[12], but they are more enriched in populations of large interneurons, which are spared, than in medium spiny projection neurons, which are selectively lost[13]. There is a good correlation, however, between the length of the CAG repeat and the density of inclusions[12–16].

Huntingtin aggregates can be labeled with antibodies to the N terminus of huntingtin or antibodies to ubiquitin, a marker for misfolded proteins, and a signal for degradation by the proteasome. Proteasomes may have difficulty digesting them, however, leading to their accumulation[17]. The aggregates contain fibers and appear to have β-sheet structure characteristic of amyloid[10], although there is controversy about whether they bind dyes that intercalate into β-SHEETS, as is a characteristic of amyloid. Other proteins, such as Creb binding protein (CBP; discussed later) containing polyglutamine may be recruited into huntingtin aggregates[18,19].

Other polyglutamine diseases. Other polyglutamine diseases, including dentato-rubral and pallido-luysian atrophy (DRPLA) and several forms of spino-cerebellar ataxia (SCA), also have intranuclear inclusions in regions roughly corresponding to the regions of neuronal degeneration[20,21]. Analysis of the mutations present in individuals with SCA1 and of unaffected individuals supports the relevance of protein aggregation to degeneration. Some individuals have been found with histidine interruptions in an expanded polyglutamine repeat in ataxin-1, the *SCA1* gene product. These histidine interruptions result in the absence of the disease and strikingly less propensity to aggregation[22].

Alzheimer's disease and tauopathies. AD is a late-onset dementing illness, with progressive loss of memory, task performance, speech, and recognition of people and objects. There is degeneration of neurons (particularly in the basal forebrain and hippocampus), but at least as important for pathogenesis may be synaptic pathology and altered neu-

Christopher A. Ross is in the Division of Neurobiology, Department of Psychiatry, and Departments of Neurology and Neuroscience, Johns Hopkins University School of Medicine, Ross Research Building, Room 618, 720 Rutland Avenue, Baltimore, Maryland 21205, USA. Michelle A. Poirier is in the Division of Neurobiology, Department of Psychiatry, Johns Hopkins University School of Medicine, Ross Research Building, Room 618, 720 Rutland Avenue, Baltimore, Maryland 21205, USA.
e-mail: caross@jhu.edu

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Table 1 Neurodegenerative diseases: proteins and pathology

Disease	Etiology	Regions most affected	Characteristic pathology	Disease proteins deposited
Huntington's disease	Huntingtin (dominant)	Striatum, other basal ganglia, cortex, other regions	Intranuclear inclusions and cytoplasmic aggregates	Huntingtin with polyglutamine expansion
Other polyglutamine diseases (DRPLA, SCA1–3, etc., SBMA)	Atrophin-1, ataxin-1–3, etc.; androgen receptor (AR) (dominant)	Basal ganglia, brain stem cerebellum, and spinal cord	Intranuclear inclusions	Atrophin-1, ataxins or AR
Alzheimer's disease (AD)	Sporadic (ApoE risk factor)	Cortex, hippocampus, basal forebrain, brain stem	Neuritic plaques and neurofibrillary tangles	Aβ peptide (from APP) and hyperphosphorylated tau
	Amyloid precursor protein (APP) (dominant)	Same as sporadic	Same as sporadic	Same as sporadic
	Presenilin 1, 2 (dominant)	Same as sporadic	Same as sporadic	Same as sporadic
Fronto-temporal dementia with Parkinsonism	Tau mutations (dominant)	Frontal and temporal cortex, hippocampus	Pick bodies	Hyperphosphorylated tau protein
Parkinson's disease (PD)	Sporadic	Substantia nigra, cortex, locus ceruleus, raphe, etc.	Lewy bodies and Lewy neurites	α-Synuclein
	α-Synuclein (dominant)	Similar to sporadic, but more widespread	Similar to sporadic	α-Synuclein
	Parkin (also DJ-1, PINK1) recessive (some dominant)	Substantia nigra	Lewy bodies absent (or much less frequent)	α-Synuclein (when present)
Amyotrophic lateral sclerosis (ALS)	Sporadic	Spinal motor neurons and motor cortex	Bonina bodies and axonal spheroids	Unknown (neurofilaments)
	Superoxide dismutase-1 (dominant)	Same as sporadic	Same	Unknown
Prion diseases (kuru, CJD, GSS disease, fatal familial insomnia, new variant CJD)	Sporadic, genetic and infectious	Cortex, thalamus, brain stem, cerebellum, other areas	Spongiform degeneration, amyloid, other aggregates	Prion protein

ApoE, apolipoprotein E; APP, amyloid precursor protein; CJD, Creutzfeldt–Jakob disease; DRPLA, dentato-rubral and pallido-Luysian atrophy; GSS, Gerstmann–Straussler–Scheinker; SBMA, spinal and bulbar muscular atrophy; SCA, spino-cerebellar ataxia.

ronal connections[23,24]. AD involves two major kinds of protein aggregates. Extracellular aggregates known as neuritic plaques have as their major constituent the Aβ peptide, which is derived from proteolytic processing of the amyloid precursor protein (APP). The Aβ-containing aggregates have β sheet structure and Congo red and thioflavin-T reactivity characteristic of amyloid[25]. There are also intracellular aggregates of the microtubule associated protein tau, called neurofibrillary tangles. The pathogenesis of AD has been greatly clarified by the identification of genetic mutations responsible for rare familial forms of the disease. These mutations are in APP itself and also in the presenilins, which are involved with the cleavage of APP (refs. 26,27). In addition, tauopathies such as fronto-temporal dementia with parkinsonism can be caused by mutations in the tau protein[28,29].

Parkinson's disease. PD is characterized by resting tremor, rigidity, slow movements and other features such as postural and autonomic instability. It is caused by degeneration of dopaminergic neurons in the substantia nigra of the midbrain and other monoaminergic neurons in the brain stem[30]. The discovery of several genes in which mutations cause early-onset forms of PD has greatly accelerated research progress[31]. Point mutations or increased gene dosage of the α-synuclein gene cause autosomal dominant PD via a gain-of-function mechanism. Recessive early-onset PD can be caused by mutations in the genes encoding parkin, DJ-1 or PINK1[32], presumably by a loss-of-function mechanism. The pathological hallmark of adult-onset PD is the Lewy body, an inclusion body found in the cytoplasm of neurons, often near the nucleus. Lewy bodies are densest in the substantial nigra but can also be present in monoaminergic, cerebral cortical and other neurons. There are also aggregates in neurites, which are referred to as Lewy neurites. A major constituent of Lewy bodies is aggregated α-synuclein protein. Lewy bodies can also be labeled for ubiquitin, a synuclein interactor termed synphilin-1, proteasome proteins, and cytoskeletal and other proteins.

Amytrophic lateral sclerosis. ALS is a progressive fatal disease caused by degeneration of lower motor neurons in the lateral horn of the spinal cord and upper motor neurons of the cerebral cortex, resulting in progressive motor weakness[33]. Rare early-onset familial forms of the disorder can be caused by mutations in the superoxide dismutase (*SOD1*) gene. The pathology does not seem to be due to alteration of SOD1 enzyme activity. Transgenic mice overexpressing mutant SOD1 have cytoplasmic inclusions containing aggregates of SOD1 protein[34,35]. Ubiquitinated aggregates are present in patient brains, although SOD1 is not usually detected in sporadic cases, and SOD1 does not usually form fibrillar structures *in vitro*.

Prion disease. Neurodegenerative diseases caused by prions can be sporadic or can be acquired either by environmental transmission or via genetic mutations[36]. Environmental pathways include eating prion particles derived from infected brain tissue or surgical implantation via infected instruments. Prion disease can also be caused by point mutations in the prion gene, leading to alterations of the prion protein. Pathology can include amyloid plaques that appear similar to those of AD and that can be labeled with prion antibodies. Prion

disease is a prototypical protein conformation disease, in that highly sophisticated studies have shown that it is caused by abnormal protein structure and not an infective viral agent. Mechanisms of prion disease have been illuminated by the discovery of prionlike protein conformational changes in yeast[37,38]. In all cases, disease is caused by abnormally folded prion proteins. Prion aggregation can take place both extracellularly and intracellularly[39,40].

Commonalities of amyloid structure

Amyloid fibrils are filamentous structures with a width of ~10 nm and a length of 0.1–10 μm. A defining feature, originally revealed by X-RAY FIBER DIFFRACTION analysis[41,42], is the presence of cross-β structure. In this structural motif, ribbonlike β-sheets are formed by β-strands running nearly perpendicular to the long axis of the fibril and hydrogen bonds that run nearly parallel to the long axis.

The most extensively characterized amyloid fibril is that formed from the β-amyloid (Aβ) peptide implicated in AD. Using SOLID-STATE NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY, the in-register, parallel β-sheet organization of fibrils formed by $A\beta_{10-35}$, a fragment of the full-length 42-residue Aβ peptide, was first described[43]. It was subsequently found that full length $A\beta_{1-42}$ forms β-sheets with the same registry and orientation[44]. Using ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY, a similar structural model was obtained for Aβ peptide and $A\beta_{40}$ (ref. 45).

A similar analysis of fibrils formed by α-synuclein found an in-register, parallel β-sheet organization[46]. The core structure of Aβ, α-synuclein and polyglutamine aggregates appears to involve both β-strands and β-turns[47–50]. Recent data suggest that the structure of polyglutamine aggregates may involve a compact β-sheet with interspersed β-turns every nine glutamines[50–52]. Thus a β-sheet plus β-turn structure may be a common form of neurodegenerative disease-related amyloid (see Fig. 2)

Consistent with a common structure, conformation-specific antibodies can bind to the amyloid fibril state of the Aβ peptide but not to its soluble monomeric state. They also bind to amyloid fibrils and amyloid-like aggregates derived from other proteins of unrelated sequence including polyglutamine, but not to non-native globular protein aggregates such as collagen, gelatin or elastin[53]. Thus, whereas there are still many unknowns regarding the detailed structure of amyloid and particularly regarding its assembly, there seem to be considerable similarities among the structures of different kinds of disease-related amyloid.

Initiation of aggregation

Neurodegenerative disease proteins often appear to be natively unfolded[54]. There may be several kinds of aggregates, including disordered or 'amorphous' aggregates, but amyloid fibrils are most characteristic. What might initiate the aggregation process?

The initiation of misfolding in a particular cell may be a stochastic event, with a constant risk over the life of the individual[55]. Amyloid formation may proceed via a process of 'seeded polymerization'[56–59]. The likelihood of aggregation could be increased by increasing protin concentration. This can be caused by genetic dosage alterations. For instance, familial PD can be caused by triplication at the α-synuclein locus[60]. Early deposition of Aβ plaques occurs in individuals with Down's syndrome, who carry an extra copy of the *APP* locus on chromosome 21. Polymorphisms in promoter sites of disease-associated genes may increase transcription and thus protein amounts, increasing the risk for neurodegenerative disease[61]. In the case of protein-coding mutations, the altered primary structure presumably makes the protein more prone to aggregate. For polyglutamine proteins, there is a very clear correlation between the expansion of the polyglutamine stretch and the aggregation of polyglutamine itself.

Covalent modifications of proteins may facilitate aggregation. Sporadic neurodegenerative diseases are generally associated with aging, which is accompanied by oxidative modifications of proteins. Oxidative modification of α-synuclein via dopamine adducts may facilitate aggregation[62]. Aging may also decrease the ability of the cell to clear misfolded

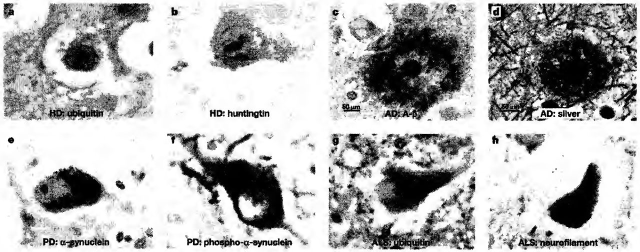

Figure 1 Characteristic neurodegenerative disease neuropathological lesions involve deposition of abnormal proteins, which can be intranuclear, cytoplasmic or extracellular. All are labeled with antibodies (except **d**), as indicated. (**a**) HD, intranuclear inclusion labeled for ubiquitin (cerebral cortex) (**b**) HD, intranuclear inclusion labeled for huntingtin (cerebral cortex). (**c**) AD, neuritic plaque labeled for Aβ (cerebral cortex). (**d**) AD, neuritic plaque, silver stained (Hirano method). (e) PD, Lewy bodies labeled for α-synuclein (fine granular brown label in this and the next panel represent neuromelanin) (substantial nigra). (**f**) PD, Lewy body labeled for phosphorylated α-synuclein (substantia nigra). (**g**) ALS, cytoplasmic skein of neurofilaments labeled with ubiquitin (medulla oblongata). (**h**) ALS, cytoplasmic skein of neurofilaments labeled with neurofilament (medulla oblongata). (Figure courtesy of O. Pletnikova and J.C. Troncoso, Division of Neuropathology, Johns Hopkins University.)

a

b

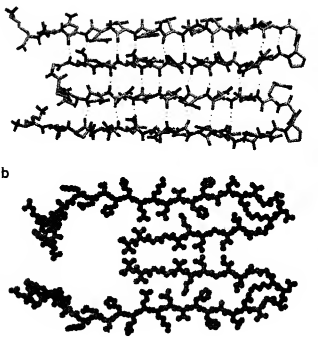

Figure 2 β-sheet, β-turn models for expanded polyglutamine and Aβ amyloid suggest commonalities in amyloid structure in different neurodegenerative diseases. (a) Sketch of expanded polyglutamine, with β-turns constrained by proline-glycine insertions every nine glutamines. This is proposed to be similar to the structure of expanded pure polyglutamine (ref. 50), in which side chains may participate in the hydrogen bonding. Light blue, carbon; dark blue, nitrogen; red, oxygen. Figure reprinted from ref. 51 with permission. (b) Model for an Aβ(1–40) fibril with β sheets formed by residues 12–24 and 30–40. Residue side chains: green, hydrophobic; magenta, polar; blue, positive; red, negative. Figure reprinted from ref. 125 with permission.

proteins. Nitration of α-synuclein has also been described[63], although whether this is an early or later event is not certain.

Another important covalent promoter of aggregation is phosphorylation. α-Synuclein purified from Lewy bodies is extensively phosphorylated on Ser129 (refs. 64–67), and experiments in cell culture suggest that Ser129 phosphorylation of α-synuclein strongly modulates interactions between α-synuclein and synphilin-1, and formation of inclusions. Thus, phosphorylation at Ser129 may have a role in the formation of Lewy bodies in PD.

Phosphorylation also is involved in aggregation of ataxin-1, the *SCA1* gene product. Elimination of a phosphorylation site in ataxin-1 markedly reduced the extent of the behavioral phenotype, inclusion formation and degeneration of Purkinje neurons in the cerebellum in fly and mouse models of *SCA-1* (ref. 68). Phosphorylation is also implicated in AD, as a major portion of the neurofibrillary tangles consists of hyperphosphorylated tau protein.

Other covalent protein modifications may also be involved. The role of ubiquitin is described in more detail later, but a ubiquitin-like modifier termed SUMO has recently been shown to be attached at lysines in the N terminus of huntingtin very near the polyglutamine stretch. Modulations by SUMO decreased aggregation, increased nuclear localization and increased neurodegeneration in a fly model of HD[69].

Proteolytic cleavage may have a role in several of the neurodegenerative diseases, including AD. Aβ is generated by the sequential action of β-secretase and γ-secretase[26,27]. By contrast, APP can be cleaved normally into a non-amyloidogenic peptide by the combination of α-secretase and γ-secretase. When APP is intact, it has very little tendency to aggregate, but the small cleavage product Aβ has a strong tendency to aggregate. The cleavage site at which γ-secretase acts can vary by several amino acids, and $A\beta_{40}$ is less toxic, and also aggregates less, than $A\beta_{42}$.

Proteolytic cleavage may be involved in HD as well. The inclusions in HD postmortem tissue are selectively labeled with antibodies to epitopes near the N terminus[15,70]. Short N-terminal fragments containing the expanded polyglutamine repeat are substantially more toxic, in most cell and mouse models, than longer or full-length huntingtin[71–73]. Huntingtin can be cleaved by several proteases, including caspases and calpains[74,75], and an unidentified aspartyl protease[76]. The N-terminal fragment of huntingtin can undergo a conformational change and form polyglutamine aggregates[77]. Cleavage of atrophin-1, the *DRPLA* gene product, may be involved in DRPLA pathogenesis[78]. Proteolytic cleavage has also been proposed for other polyglutamine disorders.

A role for proteolytic cleavage in PD pathogenesis is less well established. Lewy bodies contain both N-terminal and C-terminal epitopes of α-synuclein, indicating the presence of full-length protein. There may be also be truncated species, however. Recent observations of a transgenic mouse model of PD suggest the existence of several truncated species of α-synuclein protein, enriched in the insoluble fraction[79]. It is conceivable that these could initiate or facilitate the aggregation process.

Intermediates in the aggregation process

It is becoming increasingly clear that protein aggregation is a complex process, involving several kinds of intermediates and resulting in different kinds of fibers or amorphous aggregates. Many of the studies to date have been done *in vitro* and may not mimic the situation in human diseases, so there is much to be learned[80,81].

Aβ aggregation intermediates and toxicity. Several soluble oligomeric intermediates (larger than dimers) of Aβ peptide variants have been described independently by several different groups. One researcher proposed that $A\beta_{42}$ and the shorter 1–40 fragment form a 'micelle' structure in solution[82]. Another group identified spheroidal structures by ATOMIC FORCE MICROSCOPY (AFM) and referred to them as Aβ 'protofibrils' (ref. 83). Finally, a third group described a globular intermediate for $A\beta_{42}$ and gave it the name ADDL, for Aβ-derived diffusable ligand[84]. For simplicity, all of these species may be termed globular or oligomeric intermediates.

Chainlike fibrils have also been detected by AFM[85,86] and electron microscopy (EM)[87] for Aβ variants. These species, referred to as protofibrils, often have a curvilinear morphology, are 4 nm in height by AFM and range between 6 and 10 nm in diameter by EM. Protofibrils are shorter than mature fibers, with a length range between 5 and 160 nm. Although the pathway of assembly is not certain, it seems that globular intermediates may polymerize further to form protofibrils[88]. The term protofibril may best be reserved for small species with an early fibril-like morphology. Protofibrils then may assemble into protofilaments and finally mature fibers (Fig. 3).

Although neuritic plaques are a hallmark of AD, there is a poor correlation between plaque density in human postmortem material and antemortem cognitive deficits[89]. Soluble Aβ intermediates have been observed in human postmortem material[90,91]. Toxicity *in vitro* has been described for both globular and protofibrillar inter-

mediates[84,88]. Injection of purified Aβ monomers and spheroids into rat hippocampus[92] *in vivo* caused a block in long-term potentiation, substantiating a role for Aβ aggregation intermediates in AD neurotoxicity.

Intermediates in α-synuclein aggregation. Several different aggregation intermediates with size and morphology similar to those for Aβ have been described for α-synuclein. The pathway of assembly for intermediate forms of α-synuclein may be complex, with globular and ringlike forms in addition to curvilinear protofibrils[93]. Polyunsaturated fatty acids were reported to promote oligomerization, suggesting that α-synuclein may aggregate via an interaction with cell membranes[94]. One proposed mechanism of toxicity is the formation of pores by ringlike intermediates[93], although this idea is based on *in vitro* studies with recombinant protein.

Polyglutamine aggregation and toxic mechanisms. Fibers and amorphous aggregates with varying morphologies can be generated *in vitro* for polyglutamine-containing peptides and proteins, suggesting that the pathway of fibrillization may be complex[95]. Recent studies suggest that globular and protofibrillar intermediates form before mature huntingtin fibers, and that these might be crucial for toxicity[77,96]. An alternative possibility might involve toxicity associated with linear addition of monomers to a nascent fibril (ref. 50). Using recombinant mutant huntingtin exon 1 N terminal fragment, one group of researchers have found that Congo red, an amyloid-binding dye, enriched the population of protofibrils, suggesting that the dye may block the aggregation pathway at an early stage[77]. Congo red administration to transgenic HD mice led to an improvement of the behavioral phenotype and prolonged survival[96]. Taken together, these data are consistent with studies suggesting a role for huntingtin intermediates in aggregate formation and toxicity, although which form might be toxic is unclear.

Polyglutamine toxicity may involve recruitment into nascent polyglutamine aggregates of other proteins containing short polyglutamine stretches. Many proteins in the cell have such regions, including transcription factors and other transcriptional regulators. CBP, a key transcriptional co-activator important for the survival of many neurons, can form aberrant interactions with huntingtin *in vitro*[97]. One potential mechanism of toxicity is that a structural change in CBP, induced by its interaction with mutant huntingtin, leads to its degradation of CBP by the proteasome[98].

A different mechanism of polyglutamine toxicity proposed by another team of researchers is through interference with the proteasome. They have shown that mutant huntingtin can inhibit the proteasome, presumably by becoming engaged with it but not cleaved[99]. In these studies, cells with visible aggregates were positive for proteasome inhibition, although one cannot rule out that microaggregates not visible by microscopy were responsible for cell toxicity.

Commonalities among soluble oligomeric intermediate species. As described earlier, aggregation intermediates have been widely observed in many of the neurodegenerative diseases. Recently, an antibody has been generated that reacts with oligomeric, but not with monomeric or fibrillar forms of polyglutamine, Aβ, α-synuclein and prion protein[100]. This antibody recognized material in postmortem AD brain tissue that was distinct from plaques, and that blocked cell toxicity by Aβ, α-synuclein and polyglutamine. The actual mechanism for this block in toxicity is uncertain, because polyglutamine and α-synuclein interact intracellularly whereas Aβ interacts extracellularly. Nevertheless, it is tempting to speculate that a common structure of soluble nonfibrillar intermediates exists for all of these molecules, and that there may be common mechanisms of pathogenesis.

Therapeutic strategies

The cell has developed mechanisms to defend against misfolded and aggregated proteins. The first line of defense involves the many molecular chaperones that aid in normal folding and also in refolding of

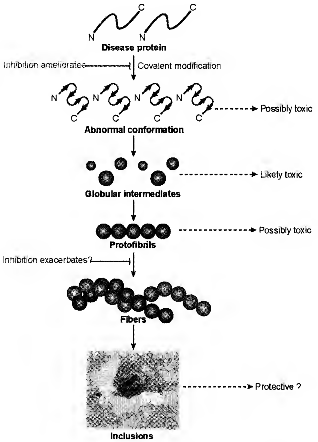

Figure 3 Flowchart for therapeutic intervention in a hypothetical several-step pathway of protein aggregation. An initiating event in aggregation may be covalent modification of the disease protein, for example by cleavage or phosphorylation, facilitating conversion of the protein to an abnormal conformation. Oligomeric (globular) intermediates may form, and then protofibrillar structures are assembled. Amyloid fibers can then form, possibly through association of protofibrillar intermediates, resulting in aggregates or inclusions visible in the light microscope. The intermediate species are hypothesized to be more toxic than either the precursor protein or the aggresomes and inclusions. Inhibition early in the pathway would be beneficial to the cell, because it may prevent the formation of potentially toxic oligomeric or other intermediates. (In a model with linear addition and no oligomeric intermediates, the process of polymerization itself would be presumed to be toxic.) By contrast, inhibition at later stages could be detrimental, because it may result in accumulation of toxic intermediates. If inhibitors could be developed that would act at the intermediate steps, they could help identify which intermediate is the toxic species. This model is based on a very hypothetical pathway for polyglutamine aggregation[51,57], and the details are subject to change; however, the concept may be generally applicable.

GLOSSARY

β-sheets A type of repetitive secondary structure that is commonly found in folded proteins. β-Sheets are formed of alternating pleated strands linked by hydrogen bonding between the amino and carboxyl groups of the peptide bond. Formation of β-sheets can be stabilized by protein oligomerization or aggregation.

X-ray fiber diffraction Technique that involves the use of X-rays to determine the quasi-atomic structure of a protein fiber. Although the wavelength of X-rays is close to the size of atoms, images are not reconstructed directly from the scattered X-rays, but from diffraction patterns. Diffraction results from the constructive and destructive interference of X-rays as they are reflected off electrons.

Solid-state NMR spectroscopy Structural method that depends on obtaining a measure of the magnetic moment of atomic nuclei, which is obtained applying an external magnetic field to a substance of interest in a constant radio frequency field. By contrast to solution-state NMR, solid-state NMR is performed on material in the solid state.

Electron paramagnetic resonance spectroscopy When an atom with an unpaired electron is placed in a magnetic field, the spin of the unpaired electron can align, either in the same direction as the field or in the opposite direction. Electron paramagnetic resonance spectroscopy is used to measure the absorption of microwave radiation that accompanies the transition between these two states.

Atomic force microscopy A form of microscopy in which a probe is mechanically tracked over a surface of interest in a series of x-y scans. The force found at each coordinate is measured with piezoelectric sensors, providing information about the chemical nature of a surface.

Autophagy Vacuolation of a portion of the cell's own cytoplasm within a membrane and its subsequent digestion after fusion with a lysosome.

abnormal conformations back to the native state[101]. If this fails, abnormal proteins can be targeted for degradation by covalent attachment of polyubiquitin followed by targeting to the proteasome and degradation[102,103]. The presence of ubiquitin, chaperones and proteasome components in inclusions presumably represents cellular defenses overwhelmed by the excessive aggregation within cells. Even the inclusions themselves are the outcome of an active process by which the cell collects irreversibly aggregated protein, translocates it to an 'aggresome' near the nucleus by active transport and attempts to eliminate it, probably by AUTOPHAGIC or other lysosomal-like processes[104–106]

One therapeutic strategy would be to enhance cellular defense mechanisms. Drugs such as geldanamycin can modulate and enhance chaperone levels[107–109]. Although geldanamycin has substantial toxicity and does not penetrate the blood-brain barrier well, other drugs may be developed. It may also be possible to stimulate proteasome activity, although this might have the danger of altering the turnover of molecules normally regulated by proteasome degradation. Although proteasomes generally work best on nonaggregated proteins, even inclusions can be cleared (by proteasomes or other mechanisms) if continued production of abnormal protein is stopped[110].

Other therapeutic interventions might directly reduce the level of abnormal protein within the cell, for instance using RNA interference[111], although its delivery would have to overcome formidable barriers of entry across the blood-brain barrier and access to neurons in the relevant region of the brain. For a disease such as PD (because the substantia nigra is relatively small), viral vectors could be directly injected. In patients with the dominant familial diseases, such as PD, ALS and AD, in which there are point mutations, it may be feasible to inactivate the mutant allele selectively[112]. Another approach, at least for diseases involving extracellular aggregates, is to use antibodies. Immunization approaches to Aβ have been tried with considerable success in animal models, but with side effects including encephalitis in humans[113–115].

Small molecules, which could be developed as drugs, may be able to target the protein misfolding pathway. Congo red binds to proteins with β-sheet structure and may alter the protein misfolding pathway[77,80] and reduce toxicity *in vivo*[96]. Chemical chaperones may be developed for blocking protein aggregation. The disaccharide trehalose[116] has recently had some success for polyglutamine disease, although at high concentration.

Small molecule agents are being developed to inhibit aggregation of Aβ[117–119], α-synuclein[79] and prions[120,121]. Small molecules can inhibit polyglutamine aggregation *in vitro*[122,123]. This has led to the development of an automated small-molecule screen for *in vitro* inhibitors of polyglutamine aggregation.

One potential danger with inhibiting one step in a several step aggregation pathway is that accumulation of a toxic intermediate could make toxicity worse (see **Fig. 3**). Nevertheless, even if all compounds do not have beneficial effects, they may prove to be powerful probes for understanding of the protein misfolding pathway. These approaches could, in principle, be applied to all the diseases. Thus, a great hope in this area is that the development of understanding and therapy for one of the diseases may have implications for the others.

Another approach involves identifying specific pathogenic mechanisms for individual diseases and developing targeted therapy. Proteolytic cleavage is an especially attractive therapeutic target, because proteolytic enzymes may be amenable to the development of high-potency small-molecule inhibitors. This is a major strategy for AD, targeting both γ-secretase and β-secretase[27,124]. There is great hope that better understanding of the pathogenic pathways will lead to rational therapeutics.

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